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Persistent Activation of NF-κB/Rel by Human T-Cell Leukemia Virus Type 1 Tax Involves Degradation of IκBβ

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Activation of the eukaryotic NF-κB/Rel transcription factors by various cytokines and mitogens is a transient event, reflecting the fact that these inducers trigger the degradation and resynthesis of the dynamic NF-κB/Rel inhibitor IκBα. However, the *tax* gene product of the human T-cell leukemia virus type 1 (HTLV-1) is known to induce the persistent nuclear expression of various NF-κB/Rel factors, especially the c-Rel proto-oncoprotein, although the underlying mechanism remains unclear. In the present study, we demonstrate that Tax induces the degradation of IκBβ, another NF-κB/Rel cytoplasmic inhibitor that differs from IκBα in signal responses. Unlike that observed with IκBα, the degradation of IκBβ is not associated with its rapid resynthesis, apparently because of the failure of Tax to stimulate IκBβ gene transcription. Thus, expression of Tax in Jurkat T cells leads to the gradual depletion of IκBβ, which is correlated with the induction of c-Rel-containing κB binding complexes. Remarkably, in the three HTLV-1-infected T-cell lines investigated, little or no detectable amount of IκBβ was found. We further demonstrate that Tax is able to override the cytoplasmic retention of c-Rel by IκBβ in transiently transfected cells. Together, these studies suggest that Tax-mediated inactivation of IκBβ may play a role in the persistent nuclear expression of c-Rel induced by HTLV-1 infection.

The human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia, an acute and often fatal T-cell malignancy (41, 61). Emerging evidence suggests the critical involvement of the virally encoded Tax protein in the initiation of the HTLV-1-induced T-cell malignancy (17, 21, 40, 42, 51, 57). Tax transactivates a large variety of cellular genes involved in T-cell activation and growth, such as those encoding interleukin-2 (IL-2) (34, 49) and the alpha chain of IL-2 receptor (IL-2Rα) (13, 25, 49). Lacking DNA binding activity, Tax appears to induce the target genes indirectly through cellular pathways (18, 50), including activation of the NF-κB/Rel family of host transcription factors (5, 12, 45).

The NF-κB/Rel factors participate in the transcriptional activation of multiple growth-related genes, including those for both IL-2 and IL-2Rα, that harbor the κB enhancer element (3, 31, 48). The NF-κB/Rel family contains various dimeric complexes composed of a set of structurally related polypeptides, including p50 (NF-κB1), p52 (NF-κB2), RelA (previously named p65), RelB, and the proto-oncoprotein c-Rel (reviewed in references 16 and 48). In resting T cells, these NF-κB/Rel complexes are sequestered in the cytoplasm as latent precursors by physical association with a family of ankyrin motif-rich inhibitory proteins (6) including IκBα (2, 19), IκBβ (58), p105 (precursor of p50 [35, 39, 44]), and p100 (precursor of p52 [35, 38, 54]). IκBα appears to play a major role in the regulation of the transient nuclear expression of the RelA/p50 NF-κB heterodimer that accompanies cellular activation by various stimuli, including mitogens like phorbol esters and cytokines such as tumor necrosis factor alpha. Such cellular activation triggers the phosphorylation and subsequent degradation of IκBα and the concomitant nuclear translocation of the NF-κB heterodimer (7, 10, 11, 20, 43, 53). However, since the nuclear NF-κB activates the expression of the IκBα gene, the depleted IκBα pool can be rapidly replenished through the enhanced de novo synthesis of IκBα protein. This autoregulatory mechanism ensures that the biological function of NF-κB is transient. In contrast to that of IκBα, IκBβ degradation occurs only in cells stimulated with certain inducers, such as the bacterial lipopolysaccharide and IL-1 (58). Moreover, since the expression of the IκBβ gene appears not to be induced by NF-κB, the degraded IκBβ protein cannot be rapidly replenished through de novo protein synthesis. Thus, degradation of IκBβ is associated with persistent nuclear expression of NF-κB/Rel (58). Up to now, it is not known what type of cell activation agents induce the degradation of IκBβ in human T cells. Similarly, neither p105 nor p100 appears to undergo rapid inducible degradation following T-cell activation (48, 54).

HTLV-1 Tax has been shown to induce the nuclear expression of a number of NF-κB/Rel species (50), although the underlying mechanism is not yet clear. Recent studies have demonstrated that Tax induces the phosphorylation and degradation of IκBα (9, 26, 27, 29, 33, 52), which may contribute to the early-phase induction of the p50/RelA heterodimer by this viral protein (26). However, since one of the characteristics of Tax-mediated activation of NF-κB/Rel is the persistent high-level nuclear expression of c-Rel-containing complexes (28, 30), additional mechanisms may exist. In this regard, recent studies have shown that Tax is able to physically associate with several NF-κB/Rel and IκB family members, which appears to correlate with the activation of NF-κB/Rel (8, 22, 23, 36, 55, 56, 59).

We demonstrate here that Tax activation of NF-κB/Rel in both HTLV-1-infected and Tax-transfected T cells is correlated with the degradation or down-regulated expression of IκBβ. We also show that Tax is able to override the cytoplasmic retention of c-Rel by IκBβ.

MATERIALS AND METHODS

Cell lines and treatments. The HTLV-1-infected T-cell lines C8166, HUT102, and K3T as well as noninfected Jurkat leukemic T-cells were maintained in

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RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. JPX-9 cells (37) were maintained in the same complete medium supplemented with 400 μg of G418 antibiotic per ml to select for expression of the neomycin resistance gene. To induce the expression of Tax, the cells were treated with 20 μM $CdCl_2$. Monkey kidney COS7 cells were cultured in Iscove's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics.

Immunoblotting and EMSA. The HTLV-1-infected T cells and noninfected parental Jurkat or JPX-9 cells were collected by centrifugation at 800 × *g* for 5 min. Subcellular protein extracts were prepared as previously described (47). For preparation of whole-cell extracts, the cells were lysed in ELB buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.0], 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.5 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride) and then vortexed for 5 min at 4°C. For immunoblotting analyses, protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with various antipeptide antisera with an enhanced chemiluminescence detection system (ECL; DuPont, NEN). Electrophoretic mobility shift assays (EMSA) were performed by incubating the nuclear extracts (~8 μg) with a ^{32}P-radiolabeled high-affinity palindromic κB probe, κB-pd (coding strand sequence was 5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′), and then resolving the DNA-protein complexes on native 5% polyacrylamide gels (4). For antibody supershift assays, 1 μl of the indicated antipeptide antisera was added to the EMSA reaction mixture 10 min prior to electrophoresis.

cDNA expression vectors and COS7 cell transfection. pCMV4HA-IκBβ was constructed by inserting three copies of the influenza virus hemagglutinin epitope tag (YPYDVPDYA) upstream of the translational initiation codon of the mouse IκBβ cDNA (58) cloned in the pCMV4 expression vector (1). Plasmid DNA was transfected into COS7 cells by the DEAE-dextran method as previously described (15).

Indirect immunofluorescence. COS7 cells were seeded on four-well chamber slides (Nunc, Naperville, Ill.) and transfected with DEAE-dextran (24). Indirect immunofluorescence assays were performed with an anti-c-Rel-specific antibody as previously described (15).

Northern (RNA) blot analysis. Total cytoplasmic RNA was isolated with an extraction reagent (TRI REAGENT; Molecular Research Center, Inc., Cincinnati, Ohio) according to the manufacturer's instructions. About 20 μg of RNA was fractionated on a 1% formaldehyde-agarose gel and then transferred onto a piece of nylon membrane. The membrane was hybridized at 42°C for 24 h with ^{32}P-labeled cDNA of human IκBα (19) or murine IκBβ (58) in a hybridization buffer (5× Denhardt's–5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.5% SDS–200 μg of denatured salmon sperm DNA per ml) supplemented with either 50% (for IκBα) or 43% (for IκBβ) formamide. The membranes were washed at 65°C in 0.2× SSC–0.1% SDS (for IκBα) or at 50°C in 2× SSC–0.1% SDS (for IκBβ) for 15 to 30 min and then subjected to autoradiography.

RESULTS

The steady level of IκBβ is strikingly low in HTLV-1-infected cells. Previous studies have demonstrated that IκBα undergoes degradation and resynthesis in HTLV-1-infected T cells (29, 52). This dynamic change of IκBα may contribute to the constitutive nuclear expression of NF-κB/Rel. However, the nuclear NF-κB/Rel in both the HTLV-1-infected and Tax-expressing T cells has been shown to be composed of primarily c-Rel-containing complexes but only residual amounts of RelA (30), a known physiological target of IκBα. It is thus suggested that other mechanisms may also be involved in the activation of NF-κB/Rel by Tax. To explore this possibility, the expression patterns of various other NF-κB/Rel inhibitors, including IκBβ, p105, and p100, were analyzed by immunoblotting with three HTLV-1-infected T-cell lines (C8166, HUT102, and K3T) and the noninfected Jurkat cell line. As previously observed (30), the whole-cell expression level of p100 as well as that of p52 in the HTLV-1-infected T cells was slightly higher than that in the noninfected Jurkat cells (Fig. 1, upper panel). In contrast, p105 appeared to be underexpressed in these virally infected cells, although the steady level of its processing product p50 was similar to that detected in Jurkat cells (Fig. 1, middle panel). Immunoblotting analysis of Jurkat cell extracts with a human IκBβ-specific antibody (IκBβ C20; Santa Cruz Biotechnology, Inc.) readily detected the 45-kDa IκBβ protein (Fig. 1, lower panel, lane 1), which did not react with either

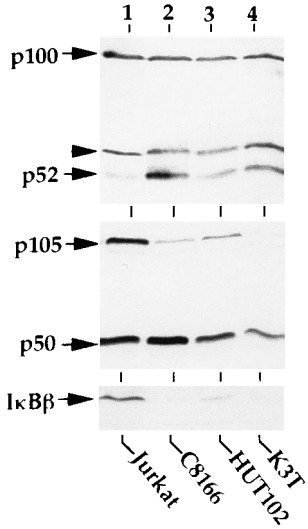

FIG. 1. Expression of IκB-like proteins in HTLV-1-infected T cells and noninfected Jurkat cells. Whole-cell extracts were isolated from either Jurkat cells or the indicated HTLV-1-infected T cells. The extracts (~15 μg) were subjected to immunoblotting analyses with peptide-specific antisera recognizing the N-terminal 21 amino acids of p100/p52 (upper panel) or p105/p50 (middle panel) or the C-terminal 20 amino acids of human IκBβ (IκBβ C-20; Santa Cruz Biotechnology, Inc.; lower panel). The arrowhead shown in the upper panel indicates a nonspecific protein band cross-reacting with anti-p100.

nonrelated immune or preimmune sera (data not shown). A similar IκBβ protein species had also been detected in a previous study (58). More importantly, the IκBβ protein was hardly detectable in the HTLV-1-infected T cells (Fig. 1, lower panel, lanes 2 to 4). Although Jurkat cells are not the most appropriate control for the HTLV-1-infected cells, the strikingly low-level expression of IκBβ in all three HTLV-1-infected T-cell lines investigated indeed suggests the possibility that HTLV-1 infection may induce the degradation of IκBβ. Furthermore, since Tax is the only viral gene product expressed in C8166 cells (46), it is likely that this specific action of HTLV-1 is mediated by the Tax protein.

Tax induces the degradation of IκBβ. To directly examine whether Tax induces the degradation of IκBβ, Jurkat T cells stably transfected with a metal-inducible *tax* cDNA construct (JPX-9; see reference 37) were used. As shown in Fig. 2A, incubation of the cells with $CdCl_2$ induced the expression of Tax (Fig. 2A, upper panel). Importantly, induction of Tax expression in these cells led to the gradual disappearance of IκBβ (Fig. 2A, lower panel, lanes 1 to 4). Furthermore, the disappearance of IκBβ appeared to be the result of Tax expression, since the IκBβ level was not significantly influenced in the parental Jurkat cells treated with $CdCl_2$ (Fig. 2A, lower panel, lanes 5 to 8). Parallel Northern blot analysis revealed that the IκBβ mRNA expression level was not appreciably affected by Tax (Fig. 2B, upper panel) although the IκBα messenger was markedly induced (lower panel). Thus, the Tax-induced disappearance of IκBβ most likely resulted from the degradation of this inhibitory protein. EMSA of the nuclear extracts isolated from these $CdCl_2$-treated JPX-9 cells demonstrated that induction of Tax expression led to the persistent nuclear expression of κB DNA binding factors (Fig. 2C, lanes 1 to 4). Furthermore, the κB binding complexes induced during the late period (48 h) of Tax induction contained not only RelA but also large amounts of c-Rel, as determined by antibody supershift assay (lanes 5 to 7). Thus, sustained nuclear

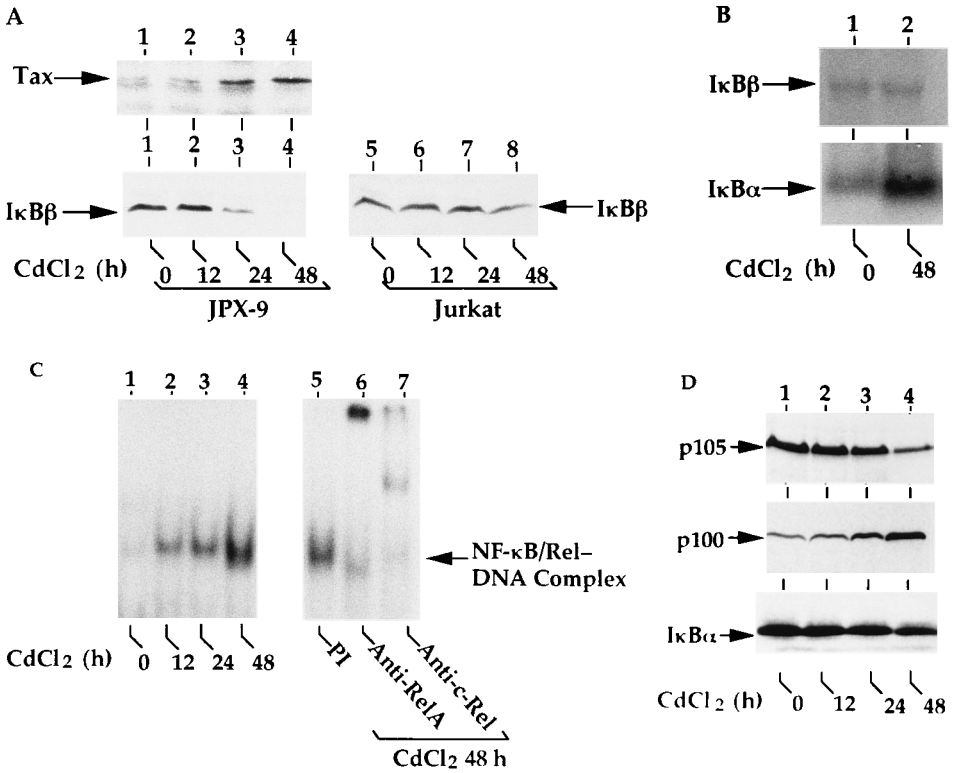

FIG. 2. Tax-induced degradation of IκBβ is associated with the nuclear expression of c-Rel-containing κB binding complexes. JPX-9 or the parental Jurkat cells were incubated with $CdCl_2$ (20 μM) for the indicated time periods and then collected for preparation of either whole-cell or nuclear extracts or total cellular RNA. (A) Whole-cell extracts isolated from either JPX-9 cells (lanes 1 to 4) or Jurkat cells (lane 5 to 8) were subjected to immunoblotting with peptide-specific antisera recognizing the C-terminal 15 amino acids of Tax (upper panel) or the C terminus of IκBβ (lower panel). (B) Total cytoplasmic RNA isolated from either nontreated (lane 1) or $CdCl_2$-treated (lane 2) JPX-9 cells was subjected to Northern blot analysis with ^{32}P-labeled cDNA of IκBβ (upper panel) or IκBα (lower panel). (C) Nuclear extracts isolated from the JPX-9 cells, treated with $CdCl_2$ for the indicated time periods, were subjected to EMSA in the absence (lanes 1 to 4) or presence (lanes 5 to 7) of either a preimmune serum (PI) or the indicated immune sera. (D) Whole-cell extracts isolated from $CdCl_2$-treated JPX-9 cells were subjected to immunoblotting with immune sera specific for p105 (upper panel), p100 (middle panel), or IκBα (lower panel).

expression of c-Rel appears to be a unique feature of Tax-mediated activation of NF-κB/Rel in both Tax-transfected and HTLV-1-infected T cells. This feature is in contrast to the transient nuclear expression of the RelA/p50 NF-κB heterodimer associated with the cytokine-induced degradation of IκBα (7).

Immunoblotting analyses of other forms of NF-κB/Rel inhibitors revealed that along with the induction of Tax the steady level of p105 was also decreased, although moderately (Fig. 2D, upper panel). As previously observed (26), p100 was even markedly induced by Tax (middle panel). However, the IκBα expression level was not significantly influenced (lower panel) even though Tax also induces the degradation of this dynamic NF-κB inhibitor (9, 26, 33, 52). This was apparently because Tax induced the expression of IκBα mRNA (Fig. 2B; also see reference 26) and the rapid de novo synthesis of IκBα protein (52).

Together, these results suggest that Tax-induced persistent nuclear expression of NF-κB/Rel, especially c-Rel, is associated with the persistent degradation of IκBβ.

Tax overrides the cytoplasmic retention of c-Rel by IκBβ. To examine whether IκBβ physically associated with c-Rel in the cytoplasm of nontreated JPX-9 cells, coimmunoprecipitation was performed with either a preimmune serum or the IκBβ-specific antiserum, and this was followed by analysis of the immunoprecipitates by immunoblotting with anti-c-Rel. As shown in Fig. 3, the 80-kDa c-Rel protein was coprecipitated by the IκBβ-specific antibody (lane 3) but not by the preimmune serum (lane 2). Since the IκBβ-specific antibody had no direct immunoreactivity with c-Rel, it thus suggested that c-Rel was precipitated through its physical interaction with IκBβ.

Using a transient-transfection model, we next investigated whether Tax was able to override the cytoplasmic retention of c-Rel by IκBβ. For these studies, COS cells were cotransfected

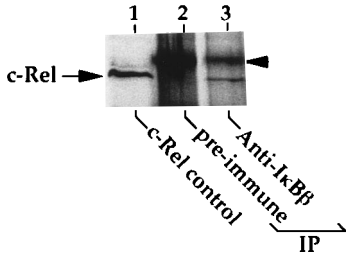

FIG. 3. Physical interaction of c-Rel with IκBβ in JPX-9 cells. Cytoplasmic extract isolated from nontreated JPX-9 cells was subjected to immunoprecipitation with either a preimmune serum (lane 2) or a peptide-specific antiserum recognizing the C terminus of human IκBβ (IκBβ C-20; Santa Cruz Biotechnology, Inc.; lane 3). The immunoprecipitates (IP; lanes 2 and 3) were then subjected to SDS-PAGE and immunoblotting with a peptide-specific antiserum recognizing the C-terminal 15 amino acids of human c-Rel (14). In lane 1, the JPX-9 cell extract was directly analyzed by immunoblotting with anti-c-Rel.

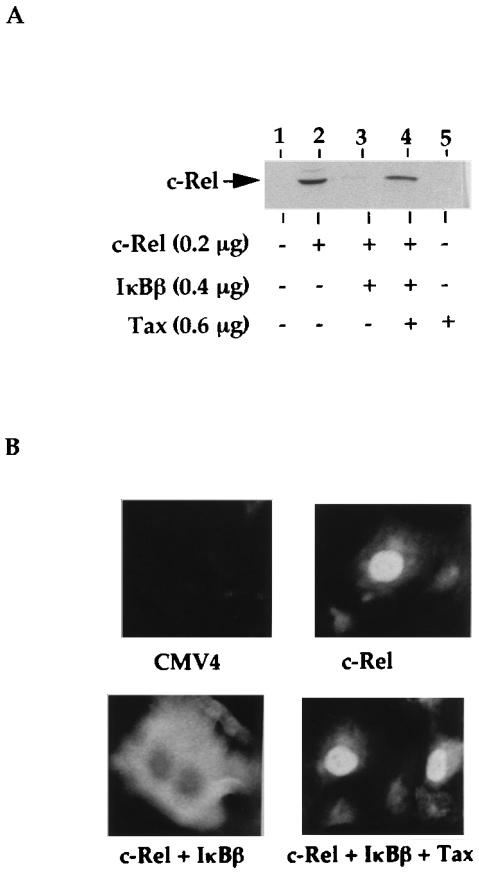

FIG. 4. Tax is able to override the IκBβ-mediated cytoplasmic retention of c-Rel in transfected COS cells. (A) COS cells were transfected with the indicated amounts of cDNA expression vectors in various combinations. Total transfected DNA was normalized with the parental vector pCMV4 lacking a cDNA insert. At about 48 h posttransfection, nuclear extracts were prepared from the recipient cells and then subjected to immunoblotting with anti-c-Rel. (B) COS cells were transfected with either a parental pCMV4 vector lacking a cDNA insert (top left), 0.2 μg of c-Rel (top right), 0.2 μg of c-Rel and 0.4 μg of IκBβ (lower left), or 0.2 μg of c-Rel, 0.4 μg of IκBβ, and 0.6 μg of Tax (lower right). The amount of the transfected DNA was adjusted with pCMV4. The subcellular localization of the transfected c-Rel was analyzed by indirect immunofluorescence assay with the c-Rel-specific antiserum.

with c-Rel and IκBβ in the presence or absence of Tax, and this was followed by immunoblotting analysis of the nuclear extracts for the expression of c-Rel (Fig. 4A). As expected, nuclear expression of c-Rel was readily detected in cells transfected with the c-*rel* cDNA expression vector (lane 2) but not in the mock-transfected cells (lane 1). Moreover, cotransfection of the cells with c-Rel and IκBβ led to the complete inhibition of the nuclear expression of c-Rel (lane 3). More importantly, expression of the Tax protein in these cells overrode the inhibitory activity of IκBβ, leading to the nuclear translocation of the sequestered c-Rel (lane 4). Of note, Tax did not induce the expression of endogenous c-Rel (lane 5) or change the expression of the transfected c-Rel, at either the RNA or the protein level (data not shown). To further confirm that Tax was able to override the cytoplasmic retention of c-Rel by IκBβ, indirect immunofluorescence was performed to examine the effect of Tax on the subcellular localization of c-Rel (Fig. 4B). As expected, the overexpressed c-Rel was primarily located in the nucleus (upper right-hand panel), and when it was coexpressed with IκBβ, this nuclear protein was sequestered in the cytoplasmic compartment because of the inhibitory action of IκBβ (lower left-hand panel). However, expression of Tax in these cells led to the relocalization of c-Rel from the cytoplasm to the nucleus (lower right-hand panel). Together, these results suggest that HTLV-1 Tax could dissociate the c-Rel/IκBβ cytoplasmic complex, leading to the nuclear translocation of c-Rel.

DISCUSSION

NF-κB/Rel transcription factors participate in the transcriptional regulation of many genes involved in cell activation and growth. The biological activity of these factors is tightly controlled by their cytoplasmic retention through physical interaction with multiple inhibitory proteins, including IκBα, IκBβ, and the NF-κB precursor proteins p105 and p100. Recent studies have suggested that these inhibitory proteins may have fundamental differences in their responses to immunological stimuli (58). In human T cells, activation of NF-κB by most inducers appears to be mediated through the degradation of IκBα, while these agents have little or no effect on the other NF-κB/Rel inhibitors (54, 58). Since the depleted IκBα can be rapidly replenished through NF-κB-induced IκBα gene expression, activation of NF-κB/Rel is normally a transient event. This autoregulatory mechanism could be important in preventing NF-κB/Rel overexpression, thus ensuring cell growth under tight control.

HTLV-1 Tax protein has been shown to induce persistent nuclear expression of a number of NF-κB/Rel species (50), which is likely a mechanism by which HTLV-1 induces the aberrant expression of various growth-related cellular genes (5, 12, 45). Kinetic studies demonstrate that Tax induces almost exclusively the p50/RelA heterodimer at early phases but induces predominantly the c-Rel-containing complexes (c-Rel/p50 or c-Rel/p52 heterodimers) at late times (26). Consistent with these studies, the c-Rel-containing complexes have also been shown to be the major κB binding complexes in HTLV-1-infected T cells that constitutively express high levels of Tax (30). Tax-mediated phosphorylation and degradation of IκBα (9, 26, 29, 52) may contribute to the early-phase induction of the RelA/p50 heterodimer, while the sustained nuclear expression of c-Rel heterodimers appears to be mediated by additional mechanisms.

In the present study, we have demonstrated that Tax induces the degradation of IκBβ, a potent cytoplasmic inhibitor of both RelA and c-Rel (58). In contrast to that observed with IκBα (26, 52), degradation of IκBβ is not associated with its rapid resynthesis, probably because the gene encoding IκBβ is not upregulated by the nuclear NF-κB/Rel (58). Remarkably, expression of Tax in T cells leads to the persistent depletion of IκBβ. This action of Tax is well correlated with the late-phase induction of c-Rel-containing κB binding complexes (Fig. 2A and C; also reference 28). The role of IκBβ degradation in the activation of c-Rel is further supported by the finding that Tax overrides the cytoplasmic sequestration of c-Rel by IκBβ. Furthermore, degradation of IκBβ appears to be associated physiologically with HTLV-1-induced T-cell abnormality. Indeed, in all three of the HTLV-1-transformed T-cell lines examined, only residual or no IκBβ protein was detected. These findings strongly suggest that degradation and down-regulated expression of IκBβ may be an important mechanism for the sustained induction of NF-κB/Rel nuclear expression by Tax.

Previous studies have shown that Tax appears to override the IκB-like activity of p105 and IκBγ (22, 23, 36, 59) and probably also of p100 (28, 36), although studies concerning p100 have been controversial (8, 60). In the present study, we observed that the p105 protein level is significantly low in all

the HTLV-1-infected cell lines analyzed, although these cells do contain normal or even larger amounts of p50, compared with the uninfected Jurkat cells. These findings could suggest that Tax probably also induces the degradation or processing of p105. Indeed, induction of Tax expression in JPX-9 cells led to a moderate decrease in levels of p105 expression.

Another feature of Tax-expressing T cells is the transcriptional induction of p100 and c-Rel (26, 30, 32, 52). We have recently found that the expression of the genes encoding both p100 and c-Rel can be potently induced by the RelA subunit of NF-κB (52, 54). Thus, it is suggested that the overall high-level expression of p100/p52 and c-Rel is likely mediated by the nuclear expression of RelA-containing NF-κB complexes, which, in turn, appears to be induced via the degradation of IκBα (26). However, the persistent nuclear expression of the active NF-κB/Rel components, especially c-Rel, may require the degradation of the cytoplasmic inhibitor IκBβ and probably also p105. Taken together, these findings suggest that activation of NF-κB/Rel by HTLV-1 Tax may be mediated by a complex mechanism involving the deregulation of various cytoplasmic inhibitors of NF-κB/Rel transcription factors.

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